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Identification of functional insulin receptors on membranes from an insulin-producing cell line (RINm5F)

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Insulin receptors on RINm5F cell membranes (an insulin-producing rat pancreatic cell line) were studied. To study the insulin receptor α-subunit, ^{125}I-labelled photoreactive insulin was covalently bound to the membranes in the absence or presence of unlabelled insulin. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under reducing conditions showed specific labelling of an M_r 130000 protein. The receptor β-subunit was studied by using a cell-free phosphorylation assay. Analysis under reducing conditions showed a phosphoprotein of M_r 95000 whose level of phosphorylation was selectively increased by insulin, and which was specifically immunoprecipitated by antibodies to the insulin receptor. Further, covalent hormone–receptor complexes purified with anti-insulin antibodies were able to undergo autophosphorylation, indicating the existence of operational receptor subunit arrangements. RINm5F cell insulin receptors (and, by analogy, possibly those of native B-cells) thus display structural and functional integrity comparable with those of conventional insulin target cells.

Insulin receptors on plasma membranes from a variety of cell types display common features thought to be essential for insulin to exert its biological effects on target cells (Kahn *et al.*, 1981; Van Obberghen, 1984). The receptor has been shown to consist of two subunits. The α-subunit (apparent M_r 130000) binds insulin, whereas the β-subunit (apparent M_r 95000) displays insulin-sensitive protein kinase activity resulting in its autophosphorylation (Kasuga *et al.*, 1982, 1983; Van Obberghen & Kowalski, 1982; Van Obberghen *et al.*, 1983; Gazzano *et al.*, 1983). Characterization of these features can thus be used as an index of the structural and functional integrity of the insulin receptors of a given cell type.

Although insulin has been shown to bind to pancreatic islets (Verspohl & Ammon, 1980) and, more specifically, to islet cells including B-cells (Patel *et al.*, 1982), the structure of the B-cell insulin receptor has not been studied. We have now shown that insulin receptors on membranes from RINm5F cells [a cloned insulin-producing cell line (Gazdar *et al.*, 1980)] are composed of both subunits, and that these subunits display the same features as attributed to them in conventional insulin target cells.

Abbreviations used: SDS, sodium dodecyl sulphate; B2-Napa-insulin, des-Phe^{B1}, B2-(2-nitro-4-azidophenylacetyl)insulin.

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Materials and methods

Materials

Triton X-100, *N*-acetyl-D-glucosamine, wheat germ agglutin–agarose, bovine serum albumin (fraction V), bacitracin and phenylmethanesulphonyl fluoride were obtained from Sigma. Adenosine 5′-[γ-^{32}P]triphosphate, tetratriethylammonium salt (5000 Ci/mmol) and Na^{125}I were purchased from Amersham International. Porcine

insulin and guinea pig anti-insulin serum were supplied by Novo Research Institute (Copenhagen, Denmark). Protein A (Pansorbin) was from Calbiochem, and reagents for SDS/polyacrylamide-gel electrophoresis were from Bio-Rad. Serum from patient B9 with antibodies to insulin receptors was a gift from Dr. C. R. Kahn (Joslin Research Laboratory, Boston, MA, U.S.A.).

Cell culture and membrane preparation

RINm5F cells were grown in culture as described previously (Praz *et al.*, 1983). The cells were harvested by scraping the plastic culture bottle surface with a rubber policeman (thereby avoiding possible membrane damage by trypsin) and suspended in 10vol. of 250mM-sucrose/5mM-Hepes containing 1.8mg of bacitracin/ml, 25mM-benzamidine, 180μg of phenylmethanesulphonyl fluoride/ml and 200 units of Trasylol (aprotinin)/ml, adjusted to pH7.0 with 1M-KOH. After homogenization (glass/glass Potter, 10 strokes, 1500rev./min), the cell material was centrifuged at 1500g (5min, 2°C) to remove nuclei and any intact cells. The pellet was resuspended by hand homogenization (Teflon/glass Potter) in 3ml of NaCl/Hepes (150mM-NaCl/30mM-Hepes, pH7.6, containing the same concentrations of proteinase inhibitors as the homogenization buffer). The supernatant was centrifuged at 24000g (15min, 2°C). This pellet was resuspended as above in 2ml of NaCl/Hepes. The 24000g supernatant was, finally, centrifuged at 100000g (1h, 2°C). The pellet was resuspended in 420μl of NaCl/Hepes, and used in all the experiments as it contained virtually all the plasma membranes.

Photolabelling of insulin receptors

The photoreactive insulin analogue B2-Napa-insulin was prepared by D. B. (Thamm *et al.*, 1980) and iodinated to a specific activity of 200–250Ci/g by using the chloramine-T method (Fehlmann *et al.*, 1982). Membranes from RINm5F cells were labelled with ^{125}I-labelled B2-Napa-insulin as previously decribed for liver membranes by Fehlmann *et al.* (1982).

Polyacrylamide-gel electrophoresis and autoradiography

The labelled proteins were analysed by one-dimensional SDS/polyacrylamide-gel electrophoresis as described by Laemmli (1970). The M_r values of the standards used were: myosin, 200000; β-galactosidase, 116000; phosphorylase *b*, 94000; bovine serum albumin, 67000; ovalbumin, 43000. The gels were stained, dried and autoradiographed as previously described (Van Obberghen *et al.*, 1981).

Results

Photoaffinity labelling of insulin receptors

When ^{125}I-labelled B2-Napa-insulin was covalently bound to membranes from RINm5F cells, SDS/polyacrylamide-gel electrophoresis of the labelled proteins under reducing conditions revealed the labelling of four proteins of M_r 180000, 130000, 67000 and 50000 (Fig. 1, lane *b*). However, only the protein with M_r 130000 was not detected in the presence of an excess of unlabelled insulin (Fig. 1, lane *a*), whereas glucagon and epidermal growth factor were without effect (results not shown). These data thus indicate that the M_r 130000 protein contains a specific insulin-binding site. Previous studies in a variety of other tissues have identified the M_r 130000 protein as the insulin receptor α-subunit (Kahn *et al.*, 1981; Van Obberghen, 1984).

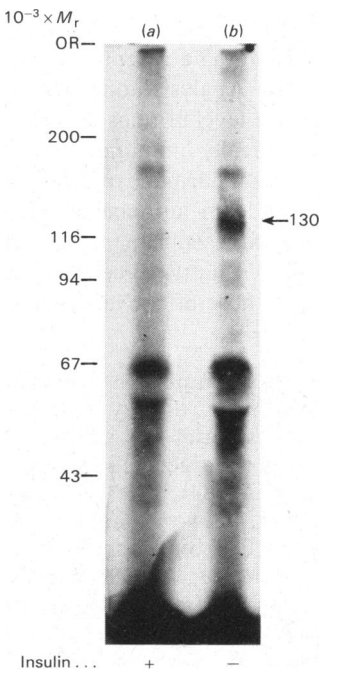

Fig. 1. *Photoaffinity labelling of insulin receptors*
Membranes purified from RINm5F cells were suspended (1mg of protein/ml) in buffer containing Hepes (50mM, pH7.6), NaCl (150mM), bovine serum albumin (10mg/ml) and bacitracin (1mM), and incubated for 90min at 15°C in the dark with ^{125}I-labelled B2-Napa-insulin (3nM) either in the absence (*b*) or presence (*a*) of unlabelled insulin (1μM). The incubation was terminated by u.v. irradiation for 5min at 4°C followed by centrifugation (10000g, 5min). The pellets were boiled in SDS solution, and the labelled proteins were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions followed by autoradiography.

Cell-free phosphorylation of partially purified insulin receptors

To investigate whether RINm5F cells possess insulin receptors which can be phosphorylated, lectin-purified glycoproteins obtained from RINm5F cell membranes were first incubated in a cell-free phosphorylation assay, and then exposed to either a normal serum or a serum containing antibodies to insulin receptor. SDS/polyacrylamide gel electrophoresis of the phosphoproteins under reducing conditions showed that anti-receptor antibodies immunoprecipitated a phosphoprotein of M_r 95000, which was not observed with a normal serum (Fig. 2, lanes *a* and *c*). Further, the phosphorylation of this M_r 95000 protein was markedly enhanced by insulin (Fig. 2, lanes *c* and *d*). This stimulating action of insulin was specific, since glucagon and epidermal growth factor were without effect (results not shown). We identify the M_r 95000 molecular species as the insulin receptor β-subunit based on its immunoprecipitation by highly specific anti-receptor antibodies, and on its electrophoretic mobility, which is identical with that previously reported for the insulin receptor β-subunit in numerous other tissues (Van Obberghen, 1984). Note that the M_r 95000 insulin receptor β-subunit appeared as a doublet. We have observed this for the insulin receptor in other tissues, and have interpreted this as the result of proteolytic degradation (Fehlmann *et al.*, 1982). The idea that the M_r 95000 phosphoprotein corresponds to the insulin receptor was further confirmed by analysis of the proteins under non-reducing conditions. Thus, when the phosphoproteins specifically immunoprecipitated by anti-receptor antibodies were separated by SDS/polyacrylamide-gel electrophoresis under non-reducing conditions, the different oligomeric forms of the insulin receptor could be seen, as

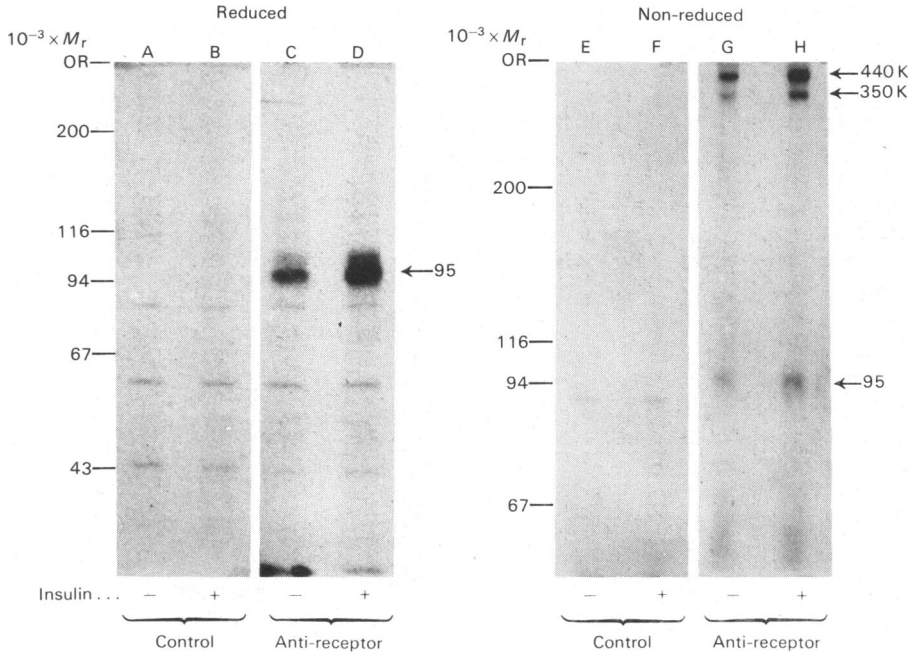

Fig. 2. *Phosphorylation of partially purified insulin receptors*

Purified RINm5F plasma membranes (1 mg of protein/ml) in Hepes buffer (50 mM, pH 7.6) containing NaCl (150 mM), bacitracin (1 mM), aprotinin (1000 trypsin-inhibitor units/ml) and phenylmethanesulphonyl fluoride (2 mM) were solubilized by Triton X-100 (1%, v/v) for 90 min at 4°C. This preparation was then centrifuged at 100000*g* for 90 min at 4°C, and the glycoproteins in the supernatant were then purified by wheat germ lectin chromatography. Aliquots of the wheat germ column eluate were incubated in the absence (*a, c, e, g*) or the presence of insulin (0.1 μM) (*b, d, f, h*) for 60 min at 20°C, and then incubated for 15 min at 20°C in a cell-free phosphorylation assay containing [γ-^{32}P]ATP (15 μM), $MgCl_2$ (8 mM) and $MnCl_2$ (4 mM). The phosphorylation reaction was terminated by addition of EDTA (25 mM) and NaF (50 mM). The samples were then exposed to either normal serum (1 : 150 dilution, or 70 μg of IgG/ml) (*a, b, e, f*) or serum containing antibodies to insulin receptor (1 : 300 dilution or 70 μg of IgG/ml) (*c, d, g, h*) for 15 h at 4°C. After precipitation by protein A, the washed pellets were analysed by one-dimensional SDS/polyacrylamide-gel electrophoresis under reducing conditions (7.5%-polyacrylamide resolving gel) or non-reducing conditions (5%-polyacrylamide resolving gel). An autoradiogram of the gel is shown.

previously described in other tissues (Chvatchko *et al.*, 1984; Van Obberghen, 1984). Indeed, two major high-M_r phosphoproteins were observed with apparent M_r values of 440000 and 350000, which probably correspond to $\alpha_2\beta_2$ and $\alpha_2\beta$ arrangements of insulin receptor subunits, respectively. In addition, we found a minor phosphoprotein of M_r 95000, which we recognized as free receptor β-subunits (Fig. 2, lane *g*). The different molecular forms of the insulin receptor were found to be hormone-responsive as insulin stimulated several-fold their phosphorylation (Fig. 2, lane *h*).

Phosphorylation of insulin receptors purified by immunoprecipitation

To investigate whether insulin receptors from RINm5F cells display an intrinsic protein kinase activity, we studied the capacity to undergo autophosphorylation in preparations of insulin receptors highly purified by two different immunoprecipitation procedures. In the first approach, insulin receptors were immunoprecipitated with antibodies to insulin receptor, then incubated with or without insulin, and finally used in a cell-free phosphorylation assay. SDS/polyacrylamide-gel electrophoresis under reducing conditions revealed that insulin receptor β-subunits isolated by anti-receptor antibodies can be phosphorylated. More importantly, insulin increased significantly the autophosphorylation of its immunoprecipitated receptor β-subunit (Fig. 3*a*). In a second approach, insulin receptors were purified from RINm5F cells by using anti-insulin antibodies, and were thereafter used in a phosphorylation assay. To this end, ^{125}I-labelled B2-Napa-insulin was covalently bound to membranes from RINm5F cells and partially purified insulin receptors were prepared from the labelled membranes by lectin chromato-

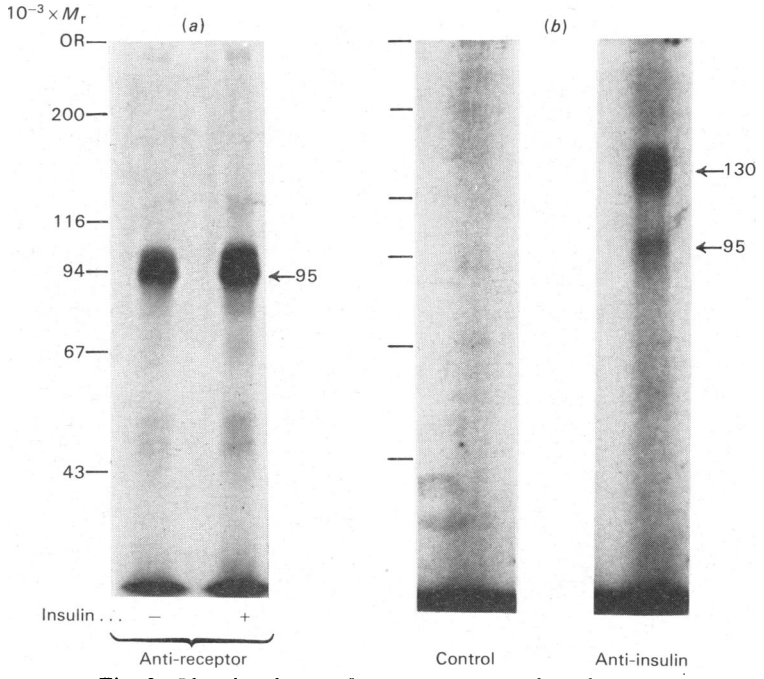

Fig. 3. *Phosphorylation of immunoprecipitated insulin receptors*

(*a*) Glycoproteins were purified from solubilised RINm5F cell membranes by wheat germ lectin chromatography as described in the legend to Fig. 2. The wheat germ column eluates were incubated with serum containing antibodies to insulin receptor (dilution 1 : 800 or 25 μg of IgG/ml). The immunoprecipitates obtained after addition of protein A were resuspended, and then incubated in the absence or presence of insulin (1 μM) for 2h at 20°C. Thereafter, the phosphorylation reaction was initiated by addition of [γ-^{32}P]ATP (15 μM), $MgCl_2$ (8mM), and $MnCl_2$ (4mM). After 20min at 20°C the reaction was stopped with boiling SDS solution, and the phosphoproteins were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions (7.5%-polyacrylamide resolving gel) followed by autoradiography. (*b*) Plasma membranes from RINm5F cells were labelled with ^{125}I-labelled B2-Napa-insulin as described in the legend to Fig. 1. The membranes were solubilized and their glycoproteins were purified by wheat germ lectin chromatography. The eluated glycoproteins were incubated for 15h at 4°C with normal serum or serum containing anti-insulin antibodies at a 1 : 100 dilution. The immunoprecipitates obtained after addition of protein A were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions followed by autoradiography.

graphy. These receptors were then exposed either to normal serum or to antibodies to insulin; the immunoprecipitates were incubated with [γ-^{32}P]-ATP and analysed by SDS/polyacrylamide-gel electrophoresis. Fig. 3(*b*) shows that anti-insulin antibodies specifically immunoprecipitated the covalent hormone–receptor complex as indicated by the presence of the M_r 130000 insulin receptor α-subunit labelled with ^{125}I-insulin. Further, this highly purified insulin–receptor complex contained intrinsic protein kinase activity, resulting in autophosphorylation of the receptor β-subunit.

Discussion

The possibility that insulin could regulate its own secretion from the pancreatic B-cell by a receptor-mediated feed-back mechanism has attracted considerable attention over the past 20 years or so. Despite numerous studies, however, performed both *in vitro* and *in vivo* in a variety of species including man, it is still not apparent as to whether such feed-back inhibition does indeed occur. There are, thus, impressive data both in favour (see, for example, Sodoyez *et al.*, 1969; Iversen & Miles, 1971; Dunbar *et al.*, 1976; Liljenquist *et al.*, 1978; De Fronzo *et al.*, 1981; Elahi *et al.*, 1982) and against (see, for example, Malaisse *et al.*, 1966; Shima *et al.*, 1977; Schatz & Pfeiffer, 1977; Marincola *et al.*, 1983) insulin affecting its own release, with additional, but more limited, data concerning other, predominantly metabolic, effects of insulin on the B-cell (Ammon & Verspohl, 1976). Perhaps the major cause of these conflicting results is the inherent difficulty associated with the study of an insulin effect on a tissue which is itself producing large quantities of the hormone. A first step towards establishing whether there is at the least the potential for insulin to influence B-cell function would be to study B-cell insulin receptors. Conventional binding studies have been performed to this end, and the results of such studies (Verspohl & Ammon, 1980), taken with morphological evidence for association of labelled insulin with the plasma membrane of B-cells (Patel *et al.*, 1982), strongly suggest the existence of insulin receptors on B-cells. These studies do not, however, indicate whether such receptors are fully operational.

The data presented here show for the first time that insulin-producing cells carry functional insulin receptors closely resembling those encountered on membranes from more conventional insulin target cells (Kahn *et al.*, 1981). Thus, when intact membranes were exposed to photoreactive labelled insulin and were then solubilized, the characteristic insulin receptor α-subunit of M_r 130000 was specifically labelled. Equally, insulin-stimulated autophosphorylation of an M_r 95000 product, which is selectively precipitable with antibody to insulin receptor, corresponds to the known behaviour of the receptor β-subunit (Kasuga *et al.*, 1983; Van Obberghen *et al.*, 1983). Further, in the present study we have used a novel double-labelling method based on the different functional properties of the insulin receptor subunits. Thus, the hormone-binding subunit, α, was probed with photoreactive insulin, whereas the protein kinase of the receptor β-subunit was labelled by autophosphorylation. The observation that the covalent hormone–receptor complex purified with insulin antibodies can undergo autophosphorylation indicates that the two receptor subunits can be isolated as a functioning structural unit. Further, this provides us with a powerful tool to unravel the mechanism of activation of the receptor kinase, which is likely to be achieved directly after hormone binding through some allosteric interaction at the level of the receptor subunits.

Characterization of the insulin receptor as reported here requires large quantities of tissue. Furthermore, for unambiguous interpretation of data, the membranes studied must be derived from a single cell type. Neither of these requirements are readily met using conventional sources of native islet cells (i.e. the isolated islet of Langerhans), which provide small numbers of cells, consisting of a mixture of the various cell types encountered in the islet. Despite recent advances in the purification of B-cells from the other pancreatic endocrine cells (Rabinovitch *et al.*, 1982; Van de Winkel *et al.*, 1982), the yield of material is still a limiting factor. To circumvent this problem, we chose to study the insulin receptors on membranes from RINm5F cells. This cloned insulin-producing cell line (Gazdar *et al.*, 1980) was originally derived from a rat insulinoma (Chick *et al.*, 1977). RINm5F cells have been shown to bind labelled insulin (Bathena *et al.*, 1982), and to release the hormone in response to a variety of stimuli (Praz *et al.*, 1983). As such, they are an attractive model of B-cell function, providing a rich source of homogeneous material for receptor studies. It is evident, however, that a rapidly proliferating cell line may differ from the native parent cell in a number of respects (Halban *et al.*, 1983), not least in its membrane properties. The data presented here must therefore be taken as an indication of the possible structure/function of native B-cell insulin receptors, and not as proof of their existence, thereby paving the way for the technically more demanding studies now required on the B-cell itself.

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